Pigs expressing salivary phytase produce low-phosphorus manure

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To address the problem of manure-based environmental pollution in the pork industry, we have developed the phytase transgenic pig. The saliva of these pigs contains the enzyme phytase, which allows the pigs to digest the phosphorus in phytate, the most abundant source of phosphorus in the pig diet. Without this enzyme, phytate phosphorus passes undigested into manure to become the single most important manure pollutant of pork production. We show here that salivary phytase provides essentially complete digestion of dietary phytate phosphorus, relieves the requirement for inorganic phosphate supplements, and reduces fecal phosphorus output by up to 75%. These pigs offer a unique biological approach to the management of phosphorus nutrition and environmental pollution in the pork industry.

The main challenge for agriculture in this century is to sustain and increase food production without degrading the environment[1]. In agriculture, global animal phosphorus pollution is a serious and growing problem[1], and the application of manure as fertilizer to land exceeds that of inorganic fertilizer or other anthropogenic fluxes[2]. High-phosphorus manure from monogastric animals[3] such as pigs and poultry arises from the inherent inability of these animals to digest plant phytate (*myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate), which accounts for up to 80% of phosphorus in common cereal grains, oil seed meals, and by-products[4,5]. Dietary supplementation with bioavailable mineral phosphate is therefore required to achieve optimal growth of animals[6]. The traditional practice of meeting nutritional requirements through phosphorus supplements has been nutritionally successful but environmentally counterproductive. As a consequence of runoff into streams and rivers, excess phosphate from manure applied as fertilizer nourishes eutrophication of phosphate-limited ecosystems[7,8], which in turn produces algal blooms, oxygen depletion, disruption of food webs, death of fish and aquatic animals, and increased production of potent greenhouse gases[9–11].

Different strategies have been devised for reducing or eliminating the need for mineral phosphorus supplementation of the swine diet. The feeding of animal by-products such as meat meal or bone meal, which have phosphorus digestibilities up to 87% (ref. 12), or of processed food wastes, has had a long history. However, concern about the spread of animal disease[13,14] has forced a transition to plant sources of phosphorus. The feeding of low-phytate corn, which reportedly improves the bioavailability of phosphorus from 9% to 62% (ref. 15), may become an option if varieties with suitable agronomic traits can be developed[16]. The most widely practiced strategy is to supplement feed with phytase, an enzyme that releases phosphate from phytate[8]. This practice has led to reductions in fecal phosphorus reportedly as high as 56% (ref. 17).

Transgenic augmentation of the natural repertoire of digestive enzymes with phytase could in principle relieve monogastric animals from the dependence on high-value specialty feedstuffs for bioavailable phosphorus. We recently demonstrated the feasibility of this approach with transgenic mouse models, using the salivary gland to deliver the highly active, low-pH optimum, and protease-resistant *Escherichia coli* phytase into the digestive tract[18,19]. In a separate study we also demonstrated that inclusion of *E. coli* phytase in poultry diets is as efficacious as adding the commercial fungal phytase[20].

We now report the development of transgenic pigs producing salivary phytase. These pigs seem to require almost no inorganic phosphate supplementation for normal growth and excrete up to 75% less fecal phosphorus than non-transgenic pigs.

Results

Using the PSP/APPA transgene[18] (parotid secretary protein promoter linked to the *E. coli appA* phytase gene), we produced 33 transgenic founder (G_0) piglets, of which 14 produced 5 to 6000 U/ml of phytase in the saliva at 7–11 days of age. Fifteen produced less than 5 U/ml, and four lacked detectable salivary phytase activity. These 33 different lines of transgenic pigs were generated from the microinjection of 4,147 pronuclear embryos with an efficiency of 0.8%. The transgene copy numbers were 35 and 2 for lines WA and JA, respectively, the lines that have received the most study. Transgenic G_1 progeny have been obtained from 13 founder lines, and farrowings are continuing for the remaining lines. Of 6 litters sired by founder boar 167-02 of line WA, 25 of 53 piglets were transgenic. This line of transgenic pigs showed the highest phytase activity at birth of all lines. The activity of phytase produced by G_1 piglets from these farrowings ranged from 341 U/ml to greater than 10,077 U/ml with a median of approximately 2,000–3,000 U/ml, which was two- to fivefold higher than that of most other lines. It should be noted that accurate determination of phytase activity in saliva sam-

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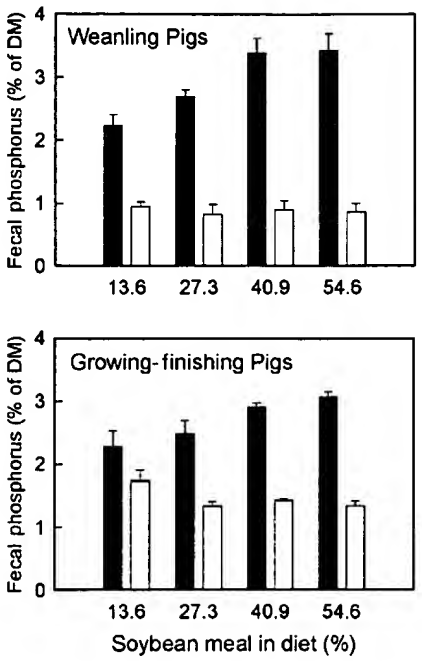

Figure 1. Total phosphorus content (on a dry matter basis) of fecal matter from non-transgenic pigs (■) and transgenic pigs (□) of line WA fed different levels of soybean meal as the sole source of dietary phosphorus. DM, Dry matter content of feces.

ples is confounded by several factors, including feed and water consumption before sampling and fluctuations in saliva production.

The presence of phytase activity in saliva indicated the potential for phytate digestion. To determine whether salivary phytase actually promotes the digestion of phosphorus from dietary phytate, we tested transgenic G_1 pigs from line WA with a median salivary phytase averaging 2,420 U/ml in nutritional trials with soybean meal containing 53% phytate phosphorous as the sole source of phosphorus. Soybean meal was chosen as the dietary source of phytate because it is a commonly used feed ingredient and it has a comparatively high concentration of phytic acid phosphorus[21]. The true digestibility of phosphorus in the test diets by both weanling and growing-finishing transgenic pigs approached 100%, compared with approximately 50% for non-transgenic pigs (Table 1). The phosphorus content of fecal matter from transgenic weanling and growing-finishing pigs fed these diets was reduced by as much as 75% and 56%, respectively, compared with that of their non-transgenic counterparts (Fig. 1). As almost all of the dietary phosphorus was digested and absorbed, the residual phosphorus in fecal matter probably arose mainly from endogenous sources. The concentration of phosphorus in fecal matter of the growing-finishing pigs was higher than that of the weanling pigs. This was probably because of higher fermentative loss of organic matter such as non-starch polysaccharides in the well-developed large intestines of the growing-finishing pigs arising from the slower rate of passage of the contents. The slightly higher content of phosphorus in the fecal material of pigs receiving the lower level of soybean meal might have arisen from lower dilution with non-digestible components of dietary soybean meal. Greater phytase inactivation caused by a lower pH of the stomach contents expected at the lowest concentration of dietary soybean meal also may have been a contributing factor.

Table 1. True phosphorus digestibility (%) of transgenic phytase pig line WA using soybean meal as the sole source of phosphorus

Pigs	Non-transgenic	Transgenic
Weanling	48.5 ± 5.4[a] (n = 16)	87.9 ± 3.4[b] (n = 14)
Growing-finishing	51.9 ± 10.3[a] (n = 16)	98.8 ± 3.4[b] (n = 14)

[a,b]Means in the same row with different superscript letters differ ($P < 0.01$). True digestibility is the percentage of total phosphorus digested and absorbed from the diets corrected for endogenous phosphorus released from the gastrointestinal tract. Data represent mean ± s.e.m., as determined by a regression analysis technique[40,42].

The digestive effect of salivary phytase was further tested by feeding G_1 transgenic finishing pigs from founder line JA a standard finishing diet not supplemented with inorganic phosphate. In this experiment, fecal phosphorus was reduced by 67% in boars (n = 7) and 64% in gilts (n = 4) compared with that in non-transgenic sibling boars and gilts fed the same ration. This difference would probably be greater had the comparison been made with non-transgenic pigs fed the standard finishing diet supplemented with inorganic phosphorus. The average (± s.e.m.) salivary phytase activities of the boars and gilts at the time of sampling were 198 ± 71 and 182 ± 48 U/ml, respectively. The growth rate expressed as days to reach 100 kg was 145.8 ± 1.8 and 145.5 ± 3.0 days for the boars and gilts, respectively, compared with a herd value of 147 days for non-transgenic pigs receiving similar rations except that they contained supplemental phosphate.

The distribution of phytase in tissues from line WA G_1 pigs was analyzed by enzymatic and immunohistochemical methods. High phytase activities were detected in the parotid, sublingual, and submaxillary salivary glands, whereas low but substantial activities were found in tissues from the fundus region of the stomach and from the duodenum (Table 2). There was substantial phytase activity in the contents of the stomach, duodenum, and ileum, but not in the contents of the cecum or colon of weanlings. The phytase activities of comparable tissues from weanling pigs differed from one another, but were higher than those of the growing-finishing pigs. The feature common to both weanling and growing-finishing pigs was the very low phytase activity in the "major" tissues, exemplified by skin, muscle, heart, and liver. Similar tissue distributions of phytase were found for lines JA and GO (data not shown). Comparable tissues from a non-transgenic pig contained no detectable phytase activity.

The distribution of immunohistochemically detectable phytase protein in various tissues of transgenic pigs (Fig. 2) corresponded to the distribution of phytase enzymatic activity (Table 2), with the parotid, sublingual, and submaxillary glands showing comparatively intense immunohistochemical staining and with no staining of muscle. Expression was consistently found in protein-producing serous cells in the acini of the salivary glands. Milk from the founder sow CA405-02 that had farrowed transgenic piglets was negative for phytase. All tissues of transgenic pigs sampled for phytase expression seemed normal by gross morphological examination and detailed histological analysis.

Phytase purified from saliva of G_0 boar WA167-02 showed both acid phosphatase and phytase activities with a specific activity for phytate hydrolysis of 1,400 U per mg protein. Although the apparent mass of purified phytase analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis was 55 kDa, the mass determined by mass spectral analysis was 50 kDa, compared with 44.7 kDa for phytase synthesized by *E. coli*[19]. The increase in mass of the salivary phytase was due to N-glycosylation, as shown by glycoprotein staining and reduction in size after treatment with N-glycosidase F (data not shown). Like the unglycosylated enzyme from *E. coli*, salivary phytase retained more than 90% of its activity after incubation with a 1,000-fold excess of pepsin at a pH of 2.5 for 6 h, but only 10% of

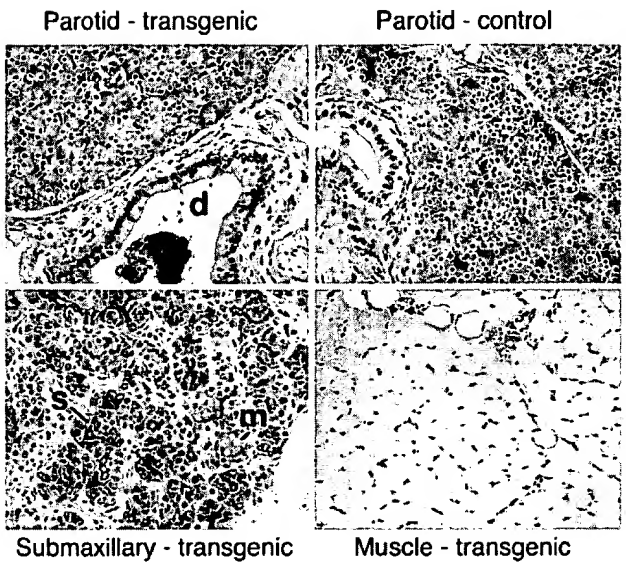

Figure 2. Tissue localization of phytase expression of line WA. Dark brown staining indicates the presence of phytase protein in acinar cells and in the parotid ducts. Left, phytase positive staining tissues; d, parotid duct; s, serous cells; m, mucous cells. Right, negative background staining in parotid gland from a non-transgenic pig and gluteal muscle from the transgenic pig.

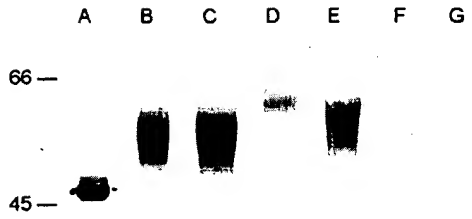

Figure 3. Western blot analysis of tissues of G_1 transgenic growing-finishing pigs from line WA. Left margin, molecular mass markers (kDa). A, purified *E. coli*-produced phytase (0.5 μg protein); B, purified salivary phytase (0.5 μg protein); C, parotid gland extract (7.5 μg protein); D, sublingual gland (15 μg protein); E, submaxillary gland (7.5 μg protein); F, fundal region of stomach tissue (15 μg protein); G, stomach contents (15 μg protein). Phytase was not detected in other tissues listed in Table 2.

its activity after incubation with a mixture of trypsin, chymotrypsin, and elastase at a pH of 7 for 6 h.

Saliva samples from 12 phytase-positive transgenic G_1 pigs from different lines were analyzed by western blotting using a monoclonal antibody against the *E. coli*-produced phytase. The antibody reacted only with the putative 55-kDa phytase (data not shown). The 55-kDa-phytase band from some pigs was smeared, indicating variation in the glycosylation of enzyme molecules. Tissue samples from growing-finishing lines WA and JA were also analyzed for phytase protein by western blotting. Phytase protein with an apparent mass of 55 kDa was detected in the saliva and parotid, sublingual, and submaxillary glands as well as in the stomach contents of a growing-finishing pig of line WA (Fig. 3), although the apparent levels in the stomach and sublingual glands were much lower than those in the parotid and submaxillary glands. The sublingual phytase seemed to have a slightly higher mass than the phytase in other tissues, which may have resulted from increased glycosylation of sublingual phytase compared with the phytase in other salivary glands—a characteristic previously reported for the mouse and rat[22,23]. The increased glycosylation may have partially blocked the monoclonal antibody-binding epitope, as less enzyme was detected in the sublingual tissue than in the submaxillary tissue despite the equal enzyme activity of the two tissues (Table 2). We obtained similar results with tissues from line JA. No immunologically reactive phytase protein was detected in other major tissues by western blotting.

Discussion

The phytase activity present in the saliva of different transgenic founder lines of pigs differed considerably. We have attributed these differences to positional effects of the transgene insertion, a phenomenom commonly seen in transgenic mice[24,25]. Because we have determined the transgene copy number for only two lines of transgenic pigs, we cannot relate copy number to phytase expression.

The salivary phytase activity and the tissue phytase activities of pigs within line WA differed between animals from the same litter and, furthermore, the phytase activities decreased with increasing age of the pig. Excluding sampling difficulties, the variation in salivary phytase activities may have arisen from random repeat-induced silencing[26], although we have no evidence for this. Likewise, the general decrease in phytase activities with increasing

Table 2. Distribution of phytase activity in tissues of G_1 transgenic pigs of line WA

Tissue[a]	Weanling pigs			Growing-finishing pigs	
	Non-transgenic[b] Sp. act.[c]	Transgenic[d] Sp. act.	Phytase % Distribution[e]	Transgenic[f] Sp. act.	Phytase % Distribution[e]
Parotid gland	0.0001	632 ± 228	100	89.2 ± 45.4	100
Sublingual gland	0.003	54 ± 23	8.6	18.0 ± 15.1	20.1
Submaxillary gland	0.001	279 ± 149	44.1	16.4 ± 16.2	12.2
Stomach	0.003	3.49 ± 1.9	0.6	0.07 ± 0.07	0.08
Stomach contents	ND	32.8 ± 9.2	5.1	0.48 ± 0.48	0.5
Duodenum	0.003	0.62 ± 0.43	0.1	0.003 ± 0.003	0.003
Duodenal contents	< 0.001	8.9 ± 2.6	1.4	< 0.001	-
Ileal contents	0.002	2.7 ± 2.7	0.4	< 0.001	-

[a]All other tissues or intestinal samples tested contained less than 0.1% of the phytase activity found in the parotid gland; these include ileum, colon, skin, brain, lung, heart, liver, pancreas, muscle, ovary, adrenal gland, spleen, testis, uterus, cecal contents, and colon contents. [b]Barrow, 9 weeks old. [c]Specific activity (Sp. act.), mmol/min per mg protein. [d]Mean ± s.e.m. for two gilts and one boar approximately 10 weeks old from line WA. [e]Normalized to the parotid gland. [f]Mean ± s.e.m. for one gilt and two boars approximately 18 weeks old from line WA. ND, not detected.

age may have been due to diminishing activity of the promoter, as found with transgenic mice (Golovan *et al.*, unpublished data), or to age-dependent silencing, which at least in mice is exacerbated by high copy number[27].

These studies provide evidence that provision of salivary phytase enables essentially complete digestion of dietary phytate phosphorus, largely relieving the requirement for inorganic phosphate supplementation, and reduces fecal phosphorus output of pigs by up to 75%. Conventional pigs require approximately 2.5 kg of supplemental dicalcium phosphate for optimal growth from weaning to market weight[6]. Transgenic pigs expressing salivary phytase can apparently recover sufficient phosphorus for optimal growth from phytate present in normal feed constituents. *E. coli* phytase degrades phytate only to inositol 2-phosphate or to inositol 5-phosphate[28]. These remaining inositol phosphate products may be further digested by other intestinal phosphatases or may be absorbed and enter the intracellular pool of inositol phosphates[29]. Despite the involvement of inositol phosphates in a variety of essential intracellular signaling processes[30], we have not detected any deleterious effect of phytase expression on the health or performance of the transgenic pigs.

The reduction in fecal phosphorus of 64–67% by finisher phytase pigs not receiving the supplemental phosphate substantially exceeds the 40% reduction reported for finisher pigs fed expensive phytase supplements (2,500 U/kg feed)[31]. A plausible reason for the greater efficiency of the salivary phytase is the much larger amount of enzyme continuously present in the stomach of the transgenic pig. A pig can secrete as much as 0.5 liters of saliva during the consumption of 0.5 kg of dry feed[32]. Consequently, pigs expressing phytase in the salivary glands may deliver as much as 200,000 U of phytase to the digestive tract during the consumption of 1 kg of feed. This compares with a typical phytase supplementation to conventional pigs of 2,500 U of phytase per kg (ref. 31). Our preliminary evidence indicates that even a modest phytase-producing line expressing 2–5 U/ml may produce sufficient phytase to satisfy the dietary phosphorus requirement. Thus, the age-dependent reduction in the amount of phytase secreted in the saliva by some lines of transgenic pigs would not have an effect on phytate digestion as long as this threshold activity is exceeded.

What is the minimum concentration of fecal phosphorus that can be attained? Because most of the dietary phosphorus is used by the transgenic pigs, as documented by the true digestibility, phosphorus present in the fecal matter of transgenic phytase pigs is probably derived from endogenous sources that escape digestion and absorption[33]. Consequently, the origins of fecal phosphorus would parallel those of endogenous nitrogenous compounds present in fecal matter[33]. It would therefore appear that we have attained nearly the maximum reduction in fecal phosphorus by digestion of dietary phosphorus, and that any further reduction would require a reduction of phosphorus released from endogenous metabolism.

Previous studies have shown a negligible effect of feeding microbial phytase on the total tract digestibility of dry matter[34,35]. However, more recent trials have documented enhanced use of dry matter[31,36]. Phytase should also abrogate the well documented anti-nutritional property of phytate, that is, the binding of essential multivalent cations, amino acids, and starch, which prevents their efficient digestion and absorption[5]. The possible benefits of phytate for the digestion of other nutrients in phytase pigs await further study.

In summary, pigs producing phytase in the saliva present a new biological approach for reducing phosphorus pollution in animal agriculture and for reducing dependence on diminishing global phosphate reserves[16,37].

Experimental protocol

Construction of the PSP/APPA transgene has been described[18]. Transgenic pigs were generated by pronuclear embryo microinjection[38]. The experimental protocols involving animals were in accordance with the Guide to the Care and Use of Experimental Animals (Vol. 1, 1980) by the Canadian Council on Animal Care. Transgenic piglets were identified at 4–11 days of age by PCR analysis of DNA from blood and tail samples, and by assay of saliva for phytase activity. For further details of the extraction conditions for genomic DNA from tail biopsies and blood, PCR conditions, and primers, see the Supplemental Text in the Web Extras page of *Nature Biotechnology* Online. One unit (U) of phytase is 1 μmol phosphate released from phytate per min. Analytical methods were essentially as described before[18,19]. Monoclonal antibodies against the purified *E. coli*-produced phytase were prepared as described before[39].

Digestion trials were done according to a 4 x 4 Latin square design as described in detail before[40]. For each trial, four 6- to 15-kg weanling pigs or 20- to 65-kg growing-finishing pigs were fed a basal diet containing soybean meal at levels of 13.6, 27.3, 40.9, and 54.6% (weight/weight) with chromic oxide as a digestibility marker (see Supplementary Tables 1 and 2 in the Web Extras page of *Nature Biotechnology* Online). The phytate phosphorus content of the soybean meal used in this study was estimated to be 53% of the total phosphorus[41].

Note: Supplementary information can be found on the Nature Biotechnology *website in Web Extras (http://biotech.nature.com/web_extras).*

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